JP-A-5-304951

(19) Japan Patent Office (JP)

(12) Japanese Unexamined Patent Application Publication (A)

(11) Patent Application Publication Number

JP-A-HEI-5-304951

(43) Publication Date: November 19, 1993

(51) Int.Cl.^{5}

C12N 5/00

Domestic classification symbol

Serial Number

E 7236-4B

FI

Technical representation place

Request for examination: Not requested

Number of claims: 12 (9 pages in total)

(21) Application Number: Japanese Patent Application HEI-4-83867

(22) Filing Date: April 6, 1992

(71) Applicant: 591052309

NT Science Co., Ltd.

2221-1, Shinmachi, Oume-shi, Tokyo

(72) Inventor: Yumi Takahashi

1279-16, Izumicho, Hachioji-shi, Tokyo

(72) Inventor: Akio Takahashi

3-4-24, Iida, Kofu-shi, Yamanashi

(72) Inventor: Kazuya Matsumoto

203, TOSOH Company House, 4-3-31, Nakanoshima, Tama-ku, Kawasaki-shi, Kanagawa

(72) Inventor: Kenji Miyata

204, TOSOH Company House, 2-2, Tsutsujigaoka, Midori-ku, Yokohama-shi, Kanagawa

(74) Agents: Masayuki Kishida, Registered Patent Attorney (and three others)

(54) [Title of the Invention] CULTURE BROTH FOR EARLY EMBRYOS AND EMBRYONIC STEM CELLS

(57) [Abstract]
[Object] Provided is a culture broth for culturing undifferentiated cells, particularly animal embryos, in unlimited animal species and/or strains, and for establishing ES cells (lines) and/or EC cells (lines) derived from animal embryos, improving the establishment efficiency, promoting their growth, and stabilizing their cultures.
[Constituent] Culturing undifferentiated cells, particularly animal embryos, and cells derived from animal embryos, with a culture broth containing insulin-like growth factor type II, or insulin-like growth factor type II and leukemia inhibitory factor, makes it possible to establish ES cells (lines) and EC cells (lines) in animal species and/or strains in which the establishment of ES cells (lines) and EC cells (lines) has been difficult to date. Also in animal species and/or strains in which ES cells (lines) and EC cells (lines) have already been established, using the same culture broth allows improving the establishment efficiency for ES cells (lines) and EC cells (lines) and stabilizing their cultures.

[Claims]
[Claim 1] An insulin-like growth factor type II having a differentiation suppressive and/or growth promotive effect on undifferentiated cells having pluripotent and/or totipotent differentiation potential.
[Claim 2] The insulin-like growth factor type II according to claim 1, having a differentiation suppressive and/or growth promotive effect on animal embryos which are undifferentiated cells.
[Claim 3] The insulin-like growth factor type II according to

claim 1, having a differentiation suppressive and/or growth promotive effect on undifferentiated cells derived from an animal embryo.

[Claim 4] The insulin-like growth factor type II according to claim 3, having a differentiation suppressive and/or growth promotive effect on undifferentiated cells, wherein the cells derived from an animal embryo are embryonal teratoma cells (lines).

[Claim 5] The insulin-like growth factor type II according to claim 3, having a differentiation suppressive and/or growth promotive effect on undifferentiated cells, wherein the cells derived from an animal embryo are embryonic stem cells (lines).

[Claim 6] A culture broth to be used to establish embryonal teratoma cells from an animal embryo in in vitro culture, comprising the insulin-like growth factor type II according to one of claims 1 to 5, or comprising, in addition thereto, a leukemia inhibitory factor as an active ingredient.

[Claim 7] A culture broth to be used to establish embryonic stem cells from an animal embryo in in vitro culture, comprising the insulin-like growth factor type II according to one of claims 1 to 5, or comprising, in addition thereto, a leukemia inhibitory factor as an active ingredient.

[Claim 8] A culture broth to be used to grow and/or maintain embryonal teratoma cells in in vitro culture, comprising the insulin-like growth factor type II according to one of claims 1 to 5, or comprising, in addition thereto, a leukemia inhibitory factor as an active ingredient.

[Claim 9] A culture broth to be used to grow and/or maintain embryonic stem cells in in vitro culture, comprising the insulin-like growth factor type II according to one of claims 1 to 5, or comprising, in addition thereto, a leukemia inhibitory factor as an active ingredient.

[Claim 10] The culture broth according to one of claims 6 to 9, wherein the insulin-like growth factor type II and/or the leukemia inhibitory factor is of the natural type.

-3-

[Claim 11] The culture broth according to one of claims 6 to 9, wherein the insulin-like growth factor type II and/or the leukemia inhibitory factor is of the recombinant type.

[Claim 12] The culture broth according to one of claims 6 to 11, wherein the insulin-like growth factor type II or the leukemia inhibitory factor comprises the full length of an amino acid sequence having the activity thereof, or comprises a fragment-length peptide having the activity thereof.

[Detailed Description of the Invention]

[0001]

[Field of Industrial Application] The present invention relates to a culture broth to be used to culture undifferentiated cells, particularly animal embryos, and to establish embryonic stem cells (lines) and/or embryonal teratoma cells (lines) derived from animal embryos, and grow and maintain embryonic stem cells (lines).

[0002]

[Background of the Invention and Prior Art] Recently, with the compilation of knowledge and the development of technology in developmental engineering and molecular biology, it has become possible to prepare transgenic animals (hereinafter TG animals) by introducing an artificially prepared extraneous gene into an early embryo, and allowing the embryo to grow to an individual (Gordon, J.W. et al., Proc. Natl. Acad. Sci. USA, 77: 7380-7384, 1980). As such, TG animals are recognized as being useful not only in investigating the functions and actions of introduced genes at the individual level by expressing genes cloned by genetic engineering techniques actually in vivo, but also in medical and industrial fields, in which disease model TG animals and TG animals that produce valuable substances have been developed.

[0003] Established methods of preparing TG animals include the microinjection method, which comprises directly injecting a fragment of an extraneous gene into an embryo in the pronucleus stage using an ultra-micropipette, the retrovirus method, which comprises infecting a retrovirus incorporating an extraneous gene

to an early embryo, and the like. However, in all of these methods, the extraneous gene is randomly incorporated in the host chromosome, and therefore it is impossible to control the site of incorporation of the extraneous gene. For this reason, these methods remain problematic with respect to reproducibility, efficient expression of the extraneous gene, and the like.

[0004] Meanwhile, as a new approach in developmental engineering, established cells that can be cultured in the undifferentiated state in vitro while retaining their potential for differentiating into various individual-forming tissues, i.e., embryonal teratoma cells (embryonal carcinoma cells; EC cells) and embryonic stem cells (embryonic stem cells; ES cells), were established. It was confirmed that an ES/EC cell, when transplanted to a normal early embryo, forms a single individual wherein cells derived from the early embryo and cells derived from the ES/EC cell are co-present, i.e., a chimeric animal (Brinster, R.L., J. Exp. Med., 140: 1949-1956, 1974). Of such chimeric animals, those having cells derived from ES/EC cells introduced to germ cells of their testis or ovary are called germline chimeras; by continuing mating with such animals as the parents, offspring essentially comprising cells derived from ES/EC cells can be obtained. This means that animals having a genetically well controlled artificial constitution can be acquired, making it possible to investigate mechanisms for development and differentiation, not only in vitro, but also at the individual level.

[0005] Research into EC cells was triggered by histological analysis of teratoma and teratocarcinoma. Because teratoma has a structure showing a morphology differentiated into a particular proper tissue or proper cells in the tumor tissue thereof, it is considered to retain a level of differentiating potential despite the tumorization of normal cells; in teratocarcinoma, it was found that vigorously growing undifferentiated stem cells are contained in similar tumor tissue. From these observations, it was suggested that by isolating teratocarcinoma-derived stem cells,

established cells capable of growing infinitely while retaining the differentiation potential may be established. Later, it was reported that by transplanting a normal blastocyst under the renal coat or to the testis, teratoma can be prepared artificially (Stevens L.C., Develop. Biol., 21: 364-382, 1970), and thereafter teratoma cells induced artificially under the renal coat were further cultured in vitro, whereby many lines of EC cells were established (Silver, L.M. et al, Teratocarcinoma Stem Cells, Cold Spring Harbor Lab., N.Y., USA., 1983). However, in the preparation of chimeric animals using EC cells, low contributions to chimera formation rate and germlines, onset of tumors postulated to be derived from EC cells in chimeric individuals and the like were pointed out (Papaioannou, V.E. et al, J. Embryol. Exp. Morph., 44: 93-104, 1978); at present, the causes thereof are considered to be due to the presence of chromosome aberrations or some gene regulatory functional abnormalities because EC cells are originally derived from a tumor.

[0006] ES cells, unlike EC cells, were established by directly culturing a normal blastocyst in vitro (Evance M.J. & Kaufman K.H., NATURE 292: 7634-7638, 1981). ES cells are also very similar to EC cells, in terms of both morphology and behavior in vitro and in vivo. However, whereas EC cells are essentially tumor cells, ES cells are for the most part normal cells retaining the karyotype of a normal diploid and have been shown to make greater contributions to both chimera formation rate and germlines (Bredley A. et al, NATURE 309: 255-256, 1986); hence, the coverage of utilization of ES cells is being widening in addition to the field of embryology.

[0007] Because ES/EC cells, like other established cells, permit the introduction of an extraneous gene using a conventional method, and also because it has become possible to select homologous recombinants only from a population of cells incorporating the extraneous gene, as described below, a feature in the preparation of TG animals, different from that of the microinjection method

and the like, was established.

[0008] In introducing an extraneous gene to cells by a conventional method, the extraneous gene is incorporated into a random site on the host chromosome, and it is known that a chromosome mutation known as homologous recombination with an endogenous gene homologous to the extraneous gene on the host chromosome at a given probability. Hence, provided that an extraneous gene having a sequence portion homologous to an intended endogenous gene is introduced, it is possible to produce a homologous recombinant which has undergone homologous recombination with the target endogenous gene sequence, simultaneously with the production of random recombinants. Also, it is possible to introduce an extraneous gene designed to select such a homologous recombinant, whereby homologous recombinants only can be selected from a population of all recombinant cells, and finally a homologous recombinant cell clone incorporating a mutation with an optionally chosen gene as the target on the host chromosome can be acquired (Mansour, S.L. et al, NATURE, 336: 348-352, 1988). Several such methods have been proposed, depending on the structure of the gene introduced, the method of selecting homologous recombinants, and the like (Capecchi, M.R., TIG, 5: 70-76, 1989), and are generally generically referred to as gene targeting. The possibility was demonstrated that by applying the gene targeting technique to the selection of homologous recombinant ES/EC cells, a homologous recombinant TG animal having an extraneous gene inserted into an optionally chosen site can be prepared, a task which has been impossible by any conventional method of preparing a TG animal, such as the microinjection method. At present, there is an increasing expectation for ES/EC cells for use in preparing TG animals via chimeric animals.

[0009] In establishing ES cells (lines) by a conventional method, fetal fibroblasts are used as a feeder cell layer, and the steps described below are performed. First, an early embryo, particularly a blastocyst or an implantation-delayed blastocyst,

is cultured on the feeder cell layer until the early embryo takes on the feeder cell layer, after which the extension growth of the trophoblasts on the outer periphery of the embryo begins. Furthermore, the inner cell mass (ICM) situated in the early embryo begins growing in a dome form on the extended trophoblasts; when the ICM has grown sufficiently, the ICM alone is separated, dispersed, and subcultured on a new feeder cell layer. Among the subcultured ICM-derived cells, a very few cells emerge which continue to grow while maintaining the undifferentiated morphology. These undifferentiated cells are further subcultured and grown, whereby ES cells (lines) are established (Robertson, E.J., Teratocarcinomas and embryonic stem cells, pp71-112, Robertson, E.J. ed., IRL Press Lim., Oxford., 1987).

[0010] As a culture broth for establishing, maintaining, and growing an ES cell strain, the DME culture broth, as the basal culture broth, supplemented with a non-essential amino acid mixture, a nucleic acid mixture, mercaptoethanol, neonatal calf serum and/or fetal calf serum, is utilized (Doetschman, T.C., J. Embryol. Exp. Morph., 87: 27-45, 1985). It was also reported that in establishing and maintaining mouse ES/EC cells (lines), differentiation suppression and growth are concurrently promoted by adding a given amount of an EC cell culture supernatant (Martin, G.R., Proc. Natl. Acad. Sci. USA, 78: 7634-7638, 1981) or a buffalo rat liver cell culture supernatant (BRL-CM) to the above-described culture broth (Smith, A.G. & Hooper, M.L., Dev. Biol., 121: 1-9, 1987), and the activity contained in these culture supernatants was named differentiation-inhibiting activity (DIA). Furthermore, later, DIA was proven to be a kind of cytokine known as leukemia inhibiting factor (LIF) (Williams, R.L. et al, NATURE, 336: 684-687, 1988). LIF improves the establishment efficiency for ES cells (lines) in a particular mouse strain, compared to the absence of LIF, and also exhibits differentiation suppressive activity and growth promotive activity on established ES/EC cells (lines) (Pease, S. et al., Dev. Biol., 144: 344-352, 1990).

-8-

However, no remarkable effect is observed in other mouse strains or other animal species. For this reason, in studies that have been conducted to date, whether or not LIF was added, the establishment of ES cells (lines) using conventional culture broth was achieved mainly in particular mouse strains such as the 129/sv strain (Handyside, A. et al, Roux's Arch. Dev. Biol., 198: 48-56, 1989) and the C57BL/6 strain (Doetschman, T.C. et al, J. Embryol. Exp. Morph., 87: 27-45, 1985); in other animal species, only a few reports are available on ES-like cells of bovines (Schellander, K. et al, Theriogenology, 31: 15-17, 1989), pigs (Strojek, R.M. et al, Theriogenology, 33: 901-914, 1990), sheep (Handyside, A., Roux's Arch. Dev. Biol., 196: 185-190, 1987), and hamsters (Doetschman, T. et al, Dev. Biol., 127: 224-227, 1988). Furthermore, it has been reported that even in ES/EC cells (lines) established in the same animal species and the same strain, differences exist among different ES/EC cell clones in terms of extraneous gene introduction efficiency, introduced gene expression efficiency, chimeric animal formation potential and the tissue distribution of cells derived from ES cells in chimeric animals, particularly in terms of the efficiency of introduction of cells derived from ES cells to germlines; it is necessary to establish a large number of ES/EC cell clones even within the same strain. Amid this situation, there is a demand for the development of a culture broth offering high establishment efficiency, and facilitating cell maintenance and growth, whatever the animal species/strain is. Insulin-like growth factor (IGF) was initially reported as a substance that exhibits insulin-like action which is not suppressed by anti-insulin antibody in human serum (Froesh E.R. et al., J. Clin. Invest., 42: 1816, 1963), and was named non-suppressible insulin-like activity soluble (NSILA-S). Furthermore, NSILA-S was purified into I and II, the amino acid sequences thereof were identified (Rinderknecht E. & Humbel R.E., J. Biol. Chem., 253: 2769, 1978). Later, because the identified amino acid sequences are similar to the amino acid sequence of insulin, and

also because insulin-like action is seen, NSILA-S was renamed IGF-I and IGF-II. Furthermore, because of similarity to insulin in terms of steric structure, IGF, along with relaxin, which is likewise similar to insulin in terms of amino acid sequence and steric structure, is classified as a member of the insulin family. In the case of humans, IGF-II is an acidic polypeptide configured with 67 amino acids, being a peptide having a molecular weight of about 7000, and having a homology of about 60% to IGF-I. It has also been shown that the IGF-II gene is localized in the short arm of chromosome No.11 in the vicinity of the insulin gene (Brissenden, J.E. et al., NATURE, 310: 781-784, 1984, Tricoli, J.V. et al., NATURE, 310: 784-786, 1984), and it has been reported that because a cDNA thereof encodes the precursor prepro-IGF-II consisting of 180 amino acid residues, IGF-II, like insulin, is synthesized as a prepropeptide hormone and undergoes processing, finally resulting in the production of IGF-II (Bell, G.I. et al., NATURE, 310: 775-777, 1984).

[0011] In addition to insulin-like action, IGF-I has been demonstrated to mediate the growth promotive action of growth hormone in vivo (Schoenle, E. et al., NATURE, 296: 252, 1982), and it has been reported that chondrocyte growth, DNA/RNA synthesis, protein synthesis, proteoglycan/collagen synthesis, and glycogen synthesis in various animals are promoted in vitro (Zapf, J. et al., Eur. J. Biochem., 87: 285, 1978). Furthermore, IGF-1 also exhibits chondrocyte colonization simulating action in agarose gel (Lindahl, A. et al., Endocrinology, 121: 1061-1069, 1987) and osteoblast growth promotive action and collagen synthesis promotive action, and has been reported to induce alkaline phosphatase activity, an osteoblast differentiation index (Zapf, J. et al., Clin. Endocrinol. Metab., 13: 3, 1984). The action of IGF-I has been thus elucidated, whereas IGF-II lacks clear action in the vivo; therefore, the physiological significance thereof remains unknown. In the rat, because serum IGF-II concentrations are elevated in the fetal stage and decrease gradually after birth,

IGF-II is considered to be the primary growth factor in the fetal stage. Also, IGF-II exhibits nearly the same action as IGF-I in vitro, but the activity thereof is lower than that of IGF-I. However, because IGF-II exhibits clear growth promotive action on cultured cells (Rechler, M.M. & Nissley, S.P., Annu. Rev. Physiol., 47: 425-442, 1985), the action thereof has been examined mainly using cultured cells. Cells that are responsive to IGF-II are prevalently fibroblasts or blood cells. Also, the NGF-like action found in pheochromocytoma, combined with a report of neurite elongation action in neuroblasts, and with the fact that the major site of production of IGF-II found in cerebrospinal fluid is considered to be the choroid plexus, also suggests the action of IGF-II in the cerebronervous system.

[0012] Meanwhile, with the advances in research into IGF receptors, it became evident that insulin and IGF-I receptors are tetramers consisting of the $\alpha2\ \beta2$ subunit structure, and have a similar structure wherein a tyrosine kinase activity site is present in an intracellular domain. However, the IGF-II receptor (hereinafter IGF-IIR) has been shown to have a totally different structure from that of the above-described receptor. That is, IGF-IIR is a single-protein receptor having a molecular weight of 220 to 270K, and not having a subunit structure, wherein the intracellular domain is short and no tyrosine kinase activity site is present. Because no enzyme activity associated with an intracellular transmission mechanism is observed in the IGF-IIR intracellular domain, it was thought in the past that IGF-IIR binds to IGF-II but does not mediate information transmission, and that all actions observed in IGF-II are due to its cross-binding to insulin and IGF-I receptor. Later, it was shown that IGF-IIR has a binding site for both IGF-II and mannose hexaphosphate (Man-6-P), and that the intracellular domain activates the Ca^{2+} channel by conjugating with the GTP-binding G protein for IGF-II. Also, because cell growth is stimulated also by activating the Ca^{2+} channel with a substance other than IGF-II, it was experimentally

- 11 -

demonstrated that the growth stimulatory action of IGF-II is caused finally by the entry of extracellular Ca^{2+} in the cells (Nishimoto, I., J. Biol. Chem., 262: 12120-12126, 1987/Ikuo Nishimoto, Tanpakushitsu, Kakusan, Koso (Protein, Nucleic Acid, Enzyme); extra issue, Saibo Zosyoku Inshi no Kiso to Rinsho, pp52-60, Nobuyoshi Shimizu, Fumimaro Takaku edt., Kyoritsu Shuppan, 1991).

[0013]

[Problems to Be Solved by the Invention] It is an object of the present invention to provide a culture broth for culturing undifferentiated cells, particularly animal embryos, in unlimited animal species and/or strains, and for establishing ES cells (lines) and/or EC cells (lines) derived from animal embryos, improving the establishment efficiency, promoting their growth, and stabilizing their cultures.

[0014]

[Means of Solving the Problems and Effect of the Invention] To accomplish the above-described object, the present inventors have developed the embodiments of the present invention described in the claims.

[0015] The IGF-II in the present invention may be derived from any animal species, and may be of the natural type purified or extracted from a naturally occurring material or the recombinant type produced by gene engineering technology. The IGF-II may also be a homologue of IGF-II in a non-human animal species, such as multiplication-stimulating activity (MSA) (Moses A.C. et al, Eur. J. Biochem., 103: 387, 1980), which is also called rat IGF-II. Furthermore, any partial peptides, mutants and modified forms based on the basic structure and/or amino acid sequence of IGF-II can also be used.

[0016] The culture broth according to the present invention comprises IGF-II or IGF-II and LIF for establishing ES/EC cells (lines) and for maintaining and growing ES/EC cells (lines). The basal culture broth for the culture broth may be any culture broth

consisting of a known composition. For example, various culture broths such as 199, NTCT135, CMRL1066, BME, MEM, DME, MB752/1, 5A, RITC80-7, F-10, F-12, L-15, and MCDB104, and culture broths based on modifications thereof can be mentioned; preferably, a culture broth of high glucose content is used, and more preferably, a culture broth of high glucose content supplemented with an additive selected from among a non-essential amino acid mixture (NEAA), a nucleic acid mixture (NMS), mercaptoethanol, a selenium compound, adrenocortical hormone and a compound thereof, transferrin, and insulin is used. To such a culture broth, neonatal calf serum (NCS) and/or fetal calf serum (FCS) is further added at 1 to 50%, preferably 5 to 30%. IGF-II is used at concentrations of 1 to 1000000 ng/ml, preferably 5 to 1000 ng/ml, more preferably 10 to 200 ng/ml; if used, LIF is used at concentrations of 1 to 1000000 unit/ml, preferably 100 to 100000 unit/ml, more preferably 1000 to 10000 unit/ml.

[0017] According to the present invention, any known culture methods can be used to culture animal embryos and cells (lines) derived from animal embryos using the culture broth. Even in animal species and/or strains for which no ES/EC cells (lines) have been established to date, the establishment of ES/EC cells (lines) becomes possible. Also in animal species and/or strains for which ES/EC cells (lines) have already been established, it is possible to improve the establishment efficiency for novel ES/EC cells (lines), stabilize subcultures, and promote their growth. Developing a culture broth that allows the easy provision of a wide variety of ES/EC cells (lines) and ES/EC cell clones as described above is expected to make significant contributions to the preparation of chimeric animals using ES/EC cells and the preparation of TG animals via chimeric animals.

[0018]

[Effect of the Invention] The present invention is effective in that it becomes possible to culture undifferentiated cells, particularly animal embryos, of conventionally limited particular

- 13 -

animal species and/or strains, in a wide variety of animal species and/or strains, and to improve the establishment efficiency for novel ES/EC cells (lines), stabilize subcultures, and promote their growth in animal species and/or strains for which ES/EC cells (lines) have already been established.

[0019]

[Examples] Examples are given below. However, the Examples below do not limit the above-described scope of claims.

[0020] Example 1:

Preparation of culture broth for early embryo and/or ES cells

With a DME culture broth of high glucose content (DME; GIBCO, 320-1965PJ) as the basal culture broth, a nucleic acid mixture (NMS) of the composition shown in Table 1, a solution of non-essential amino acids (NEAA; SIGMA, M7145), fetal calf serum (FCS; CCT), and recombinant mouse LIF (rmLIF; Wako Pure Chemical Industries, Ltd.) were added thereto in the respective amounts, and at the respective concentrations, shown in Table 2 and Table 3. Depending on the concentration of the natural type rat IGF-II added to the culture broth (nrIGF-II; SIGMA, I-2639), two kinds of culture broth were prepared: ESM/NR1 (Table 2) at 100 ng/ml and ESM/NR2 (Table 3) at 50 ng/ml.

[0021]

[Table 1]

Table 1: Composition of nucleic acid mixture

Nucleic acid	weight/pure water 100 ml
Adenosine	80 mg
Guanosine	85 mg
Cytidine	73 mg
Uridine	73 mg
Thymidine	24 mg

[0022]

[Table 2]

Table 2: Composition of ESM/NRI

Name of substance	Amount added/culture broth 100 ml	Final concentration
DME	76.5 ml	—
FCS	20.0 ml	20.0 v/v%
NEAA	1.0 ml	1.0 v/v%
NMS	1.0 ml	1.0 v/v%
nrIGF-II	1.0 ml	100 ng/ml
rmLIF	0.5 ml	5000 unit/ml

[0023]

[Table 3]

Table 3: Composition of ESM/NR2

Name of substance	Amount added/culture broth 100 ml	Final concentration
DME	77.0 ml	—
FCS	20.0 ml	20.0 v/v%
NEAA	1.0 ml	1.0 v/v%
NMS	1.0 ml	1.0 v/v%
nrIGF-II	0.5 ml	50 ng/ml
rmLIF	0.5 ml	5000 unit/ml

[0024] Example 2:

Preparation of feeder cell layers

A fetus at 15 days of gestation obtained by mating a Wistar rat
and an ACI rat was extirpated, shredded in a test tube, and
digested and dispersed by trypsin-EDTA treatment, after which the
cells were suspended in a DME culture broth supplemented with 10%
neonatal calf serum (NCS; GIBCO). Next, the cell suspension was
dispensed to a plastic dish, and cultured at 37°C in the presence
of 5% CO_2 for 1 hour. After 1 hour of cultivation, the dish was
washed with Ca^{2+}/Mg^{2+}-free Dulbecco modified phosphate buffer
solution (D-PBS (-)) to remove the suspended cells, and only the

- 15 -

fibroblasts adhering to the base were cultured.

[0025] To use the fetal fibroblasts as a feeder cell layer, mitomycin C treatment was performed. A DME culture broth supplemented with 10% NCS, and containing 10 ug/ml mitomycin C, was dispensed to the dish wherein the fibroblasts had grown sufficiently to form a single-layer sheet; after being allowed to stand at 37°C in the presence of 5% CO_2 for 2 to 3 hours, the dish was washed with D-PBS (-) three times, and then the cells were dispersed by trypsin-EDTA treatment. The cells dispersed were precipitated and recovered via centrifugation, after which they were re-suspended in a DME culture broth supplemented with 10% NCS, and this cell suspension was dispensed to the wells of a 6-well plate to obtain feeder cell layers.

[0026] Example 3:

Establishment of Wistar rat ES cell lines with ESM/NR

Wistar rats were mated, and ectopic blastocysts were obtained by uterine perfusion on day 4, counted with the day after confirmation of copulation as day 1 (Figure 1). The ectopic blastocysts were cultured on the above-described feeder cell layers using ESM/NR1, whereby growth of ICMs was observed in all the blastocysts at day 3 to 4 after initiation of the cultivation (Figure 2); the grown ICMs were separated using a micropipette and transferred onto new feeder cell layers (Figure 3). The separated ICMs were thereafter cultured using ESM/NR2; colonies showing the undifferentiated morphology were cloned 1 to 3 times, whereby a cell population consisting exclusively of cells showing the undifferentiated morphology was obtained (Figure 4).

[0027] Example 4:

Establishment of ACI rat ES cell strains with ESM/NR

In the same manner as with the above-described Wistar rats, ACI rats were mated, and blastocysts were obtained by uterine perfusion on day 4, counted with the day after confirmation of copulation as day 1. The blastocysts were cultured on the above-described feeder cell layer using ESM/NR1, whereby growth of ICMs

- 16 -

was observed in all the blastocysts at 3 to 4 days after initiation of the cultivation, and the grown ICMs were separated using a micropipette and transferred onto a new feeder cell layer. The separated ICMs were thereafter cultured using ESM/NR2, and colonies showing the undifferentiated morphology were cloned 1 to 3 times, whereby a cell population consisting exclusively of cells showing the undifferentiated morphology was obtained. The results of establishment of ACI rat ES cell strains using ESM/NR are shown in Table 4.

[0028]

[Table 4]

Table 4:

Establishment of ACI rat ES cells with ESM/NR

Number of blastocysts	Number of ICMs separated	Number of ICMs separated	Number of ES cell lines established
6	4	4	16

[0029] Example 5:

Maintenance and growth of rat ES cells with ESM/NR

Using ESM/NR2 and ESM/NR2I$^{(-)}$, which is ESM/NR2 devoid of nrIGF-II, ACI rat ES cells were cultured on feeder cell layers prepared on respective 6-well plates. On day 2 of cultivation using ESM/NR2, ES cells had grown while retaining the undifferentiated morphology (Figure 5), whereas in the cultivation using ESM/NR2I$^{(-)}$, most cells had differentiated (Figure 6). From these results of comparative cultivation, it was found that IGF-II is essential for the maintenance and growth of ES cells.

[0030] Example 6:

Differentiation induction cultivation of rat ES cells

Wistar rat ES cells established with ESM/NR were subjected to suspension culture, whereby embryoid body formation, which serves as an index of differentiation potential retention, was

investigated.

[0031] Wells with well grown undifferentiated colonies, along with feeder cell layers, were digested and disperses by trypsin-EDTA treatment; the cells were precipitated and recovered via centrifugation, and re-suspended in a DME culture broth supplemented with 10% NCS. The cell suspension was dispensed to a plastic dish and cultured at 37°C in the presence of 5% CO_2 for 1 hour, and the dish was then washed with D-PBS (-), and only non-adhering cells were recovered. The washings were centrifuged, the suspended cells were precipitated, recovered, and re-suspended in a DME culture broth supplemented with 10% NCS, and this cell suspension was dispensed to a dish for suspension culture, whereby only ES cells were subjected to suspension culture.

[0032] As a result of suspension culture of Wistar rat ES cells by the procedures described above, the cells in dispersion began aggregating on days 1 to 2 after initiation of the cultivation, and the cells differentiated into spherical hollow embryoid bodies on days 5 to 7 (Figure 7).

[Brief Description of the Drawings]

[Figure 1] Figure 1 shows ectopic blastocysts collected from a Wistar rat.

[Figure 2] Figure 2 shows an inner cell mass (ICM) growing from a blastocyst on a feeder cell layer.

[Figure 3] Figure 3 shows an inner cell mass (ICM) transferred onto a new feeder cell layer.

[Figure 4] Figure 4 shows a colony of cells with the undifferentiated morphology obtained by colony cloning.

[Figure 5] Figure 5 shows a colony with the undifferentiated morphology appearing with a culture broth supplemented with IGF-II (ESM/NR2I).

[Figure 6] Figure 6 shows a colony with a differentiated morphology appearing with a culture broth not supplemented with IGF-II (ESM/NR2I$^{(-)}$).
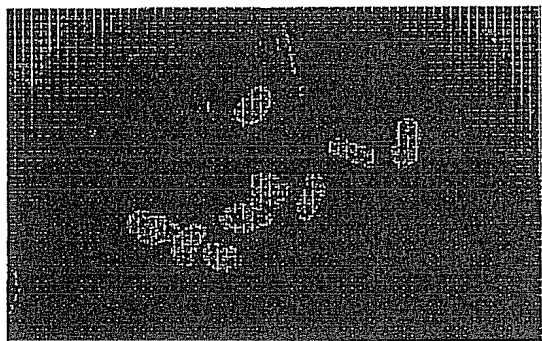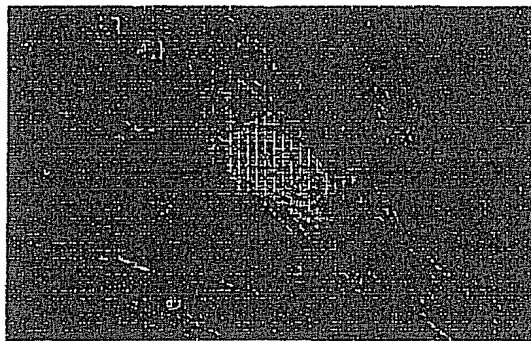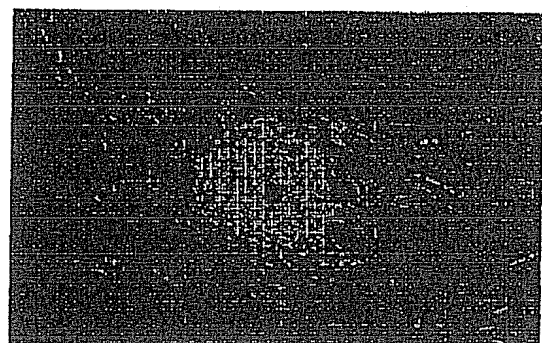
[Figure 7] Figure 7 shows embryoid bodies formed by suspension culture.
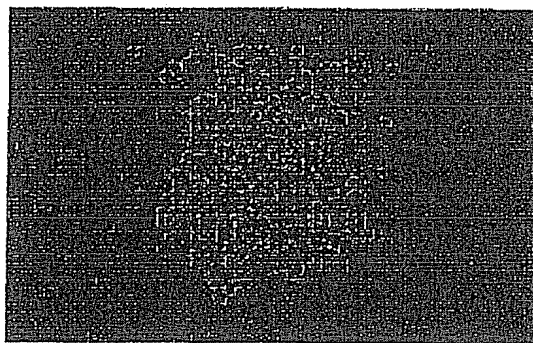
Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7